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Incorporation of farnesol significantly increases the efficacy of liposomal ciprofloxacin against *Pseudomonas aeruginosa* biofilms *in vitro*

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Abstract

The challenge of eliminating *Pseudomonas aeruginosa* infections, such as in cystic fibrosis lungs, remains unchanged due to the rapid development of antibiotic resistance. Poor drug penetration into dense *P. aeruginosa* biofilms plays a vital role in ineffective clearance of the infection. Thus, the current antibiotic therapy against *P. aeruginosa* biofilms need to be revisited and alternative anti-biofilm strategies need to be invented. Fungal quorum sensing molecule (QSM), farnesol, appear to have detrimental effects on *P. aeruginosa*. Thus, this study aimed to co-deliver naturally occurring QSM farnesol, with the antibiotic ciprofloxacin as a liposomal formulation to eradicate *P. aeruginosa* biofilms. Four different liposomes (with ciprofloxacin and farnesol: $L_{cip+far}$, with ciprofloxacin: L_{cip}, with farnesol: L_{far}, control: L_{con}) were prepared using dehydration-rehydration method and characterized. Drug entrapment and release were evaluated by spectrometry and high performance liquid chromatography (HPLC). The efficacy of liposomes was assessed using standard biofilm assay. Liposome-treated 24h *P. aeruginosa* biofilms were quantitatively assessed by XTT reduction assay and crystal violet assay, qualitatively by confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). Ciprofloxacin release from liposomes was higher when encapsulated with farnesol ($L_{cip+far}$) compared to L_{cip} (3.06% vs 1.48%) whereas farnesol release was lower when encapsulated with ciprofloxacin ($L_{cip+far}$) compared to L_{far} (1.81% vs 4.75%). The biofilm metabolism was significantly lower when treated with $L_{cip+far}$ or L_{cip} compared to free ciprofloxacin (XTT, $P<0.05$). When administered as $L_{cip+far}$, the ciprofloxacin concentration required to achieve similar biofilm inhibition was 125-fold or 10-fold lower compared to free ciprofloxacin or L_{cip} respectively ($P<0.05$). CLSM and TEM confirmed predominant biofilm disruption, greater dead cell ratio and increased depth of biofilm killing when treated with $L_{cip+far}$ compared to other liposomal preparations. Thus, co-delivery of farnesol and ciprofloxacin is likely to be a promising approach to battle antibiotic resistant *P. aeruginosa* biofilms by enhancing biofilm killing at significantly lower antibiotic doses.

Keywords: *Pseudomonas aeruginosa*, biofilm, ciprofloxacin, farnesol, liposome

Introduction

The opportunistic bacterial pathogen, *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections worldwide and ranked the second most prevalent among the Gram-negative pathogens reported to the National Nosocomial Infection Surveillance System [1]. In the USA, approximately 51,000 healthcare-associated *P. aeruginosa* infections occur annually and alarmingly, over 6,000 (13%) of these infections are caused by multidrug-resistant variant of the pathogen [2]. *P. aeruginosa* is the most commonly isolated pathogen in lung infections in cystic fibrosis patients and is considered as the leading cause for morbidity and mortality in such patients[3]. Despite aggressive therapeutic approach, current antibiotic treatments could only accomplish an adjournment of the spread of the pathogen as well as the destruction of the lung tissues; mucus-embedded biofilms persist for decades and cannot be completely eradicated. Thus, the applications of current armory of antimicrobial compounds against *P. aeruginosa* biofilm infections are needed to be revisited and alternative anti-biofilm drugs and strategies are ought to be sought [4].

P. aeruginosa biofilms are multicellular surface-attached and spatially oriented, bacterial communities encased in an extracellular matrix that display characteristic and significant resistance to antimicrobial agents and environmental stresses. Limited drug penetration through the biofilm matrix [5], nutrients and oxygen-based heterogeneity of the bacterial cell populations [6], biofilm specific bacterial phenotypes[6], subpopulations of multidrug resistant persister cells [7], and bacterial communications via quorum sensing and signal transduction systems [8] are suggested to contribute the drug resistance associated with *P. aeruginosa* biofilms. As a result of significant antibiotic tolerance, individual antibiotic therapy often fails to eliminate *P. aeruginosa* infections in CF patients and combined antibiotic therapies at high concentrations are required in managing acute CF related infections [9].

A variety of antibiotic treatment strategies such as systemic and nebulized antibiotics have been employed to treat *P. aeruginosa* biofilms in CF lungs [10-12]. However, to date, no therapeutic approach has achieved complete eradication of *P. aeruginosa* from the chronic lung infections most likely due to biofilm-specific intrinsic antibiotic resistance. In particular, the efficacy of aminoglycosides, widely used first-line therapy in the management of CF infections, is limited due to their chemical interactions with the biofilm matrix resulting slow and incomplete drug penetration into the bacterial biofilm core [13]. Conversely, Low pH in the biofilm matrix facilitates antibacterial agents such as ciprofloxacin to strongly bind to the alginates within the biofilm causing significant

reduction in the effective drug concentrations at the target site. Alarmingly, antibiotic doses below Minimum Inhibitory Concentrations (MIC) i.e. sub-MICs of tobramycin have recently been found to promote *P. aeruginosa* biofilm formation, and to generate drug resistant bacterial strains [14, 15].

Encapsulation of the drugs in to liposomes was recently introduced in overcoming nonspecific drug-binding to extracellular matrix and the metabolic protection in the biofilm and appears greatly to aid in delivering the drug of interest to the target site. For instance, liposomal amikacin demonstrated a high drug loading, long term stability, slow and sustained drug release, and a greater potential for *in vitro* penetration through mucus to reach the biofilm cells of *P. aeruginosa* [16]. Furthermore, drug delivery via liposomes potentially aids persister cell eradication due to slow and sustained release of the drug providing long term localized high concentrations of the antibiotic compared to free form of the drug in order to kill persister cells [17, 18]. .

In recent studies, a 12-carbon sesquiterpene; farnesol, a known virulence factor and repressor of yeast to hyphae morphological transition in *Candida albicans* [19-25] , was shown to inhibit the synthesis of *P. aeruginosa* quorum sensing molecule (QSM), 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal: PQS), PQS-regulated virulence factor pyocyanin [26], and bacterial swarming motility [27]. Importantly, PQS is shown to play an important role in the biofilm formation of *P. aeruginosa* [28]. Hence, we speculate that farnesol possesses inhibitory effects on *P. aeruginosa* biofilm development.

Quorum sensing has not been targeted before in the management of CF lung infections, and co-delivery of antibiotics and QSMs has never been attempted. Thus, the aim of this study was to co-deliver a naturally occurring fungal QSM (farnesol), and a well-established antipseudomonal antibiotic (ciprofloxacin), in the form of liposomes to *P. aeruginosa* biofilms. We hypothesized that a liposomal delivery system of farnesol with ciprofloxacin will efficiently disrupt and eliminate *P. aeruginosa* biofilms by targeting multiple mechanisms of biofilm development and maintenance compared to antibiotic treatment alone.

Material and methods

Preparation of liposomes

For each experiment, 4 different formulations of liposomes were freshly prepared. These included: liposome only (L_{con}), Liposome with ciprofloxacin (L_{cip}), liposomes with farnesol (L_{far}) and liposome

with ciprofloxacin and farnesol ($L_{cip+far}$). Liposomes were prepared as described by Lagace *et al* with modifications [29]. DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, Genzyme Pharmaceuticals, Switzerland) and cholesterol were added 4:1 molar ratio and dissolved in chloroform (HPLC grade, Fisher Scientific, USA) in a 1:1 w/v ratio. Farnesol 2µl (0.879g/ml density) was added to the dissolved lipid solution and mixed thoroughly for L_{far} and $L_{cip+far}$ preparations. The lipid solutions were evaporated to form a thin layer of lipid using a rotary evaporator (Buchi R-210 rotovapor, Buchi Labortechnik, Switzerland). The flasks were stored under a vacuum for an hour to remove any residual chloroform.

Ten milliliters of unbuffered saline (for L_{con} and L_{far}) or 10ml of 3mg/ml ciprofloxacin dissolved in unbuffered saline (for L_{cip} and $L_{cip+far}$, Ciprofloxacin hydrochloride USP grade, Letco Medical, Decatur, USA, Catalog No. 690953) were added to the flasks and sonicated in a water bath for 5 min. The formed multi lamellar liposomes were collected, centrifuged at 15000rpm for 10min at 4^0C. The supernatant was collected and the pellet was resuspended in unbuffered saline, washed and washes were collected. The resulting liposome pellet was resuspended in unbuffered saline as necessary and used for liposome characterization and microbiological studies.

Determination of drug entrapment efficiency

Ciprofloxacin entrapment

To determine the entrapped ciprofloxacin, the optical density of the supernatant and washes collected during the preparation of liposomes was read in a spectrophotometer (Infinite M200, Tecan Systems Inc., CA, USA) at 272nm. The entrapment efficiency (EE) was calculated using following formula;

$$\frac{\text{(Total quantity of ciprofloxacin added} - \text{the quantity of unbound ciprofloxacin)}}{\text{(Total quantity of ciprofloxacin added)}} \times 100$$

Farnesol entrapment

The entrapment of farnesol was quantified using High Performance Liquid Chromatography (HPLC) as described by Chen *et al* 2009 [30]. Supernatant and wash were filtered through 0.22µm sterile filters before performing HPLC. The assay was performed on a Waters 2495 HPLC system with a C18 reverse phase column (µBondapak®, 3.9×300mrn column, Waters Corporation, MA, USA). The

following conditions were used in the HPLC assay: mobile phase Acetonitrile: water (80:20 V/V); the flow rate 1ml/min; UV detection at 210nm; retention time 6min. All samples were analyzed in duplicates.

Farnesol entrapment was calculated as follows;

$$\frac{\text{(Total quantity of farnesol added} - \text{the quantity of unbound farnesol)}}{\text{(Total quantity of farnesol added)}} \times 100$$

Characterization of liposomes

Particle size and zeta potential

The size, polydispersity index and the zeta potential of liposomes were determined by Dynamic Light Scattering Zetasizer Nano ZS (Malvern instruments Ltd, UK).

Morphology of liposomes

The morphology of liposomes was visualized by transmission light microscope (TLM, Leica TCS SP5, Leica Microsystems, IL) fluorescent microscopy (FM, Stained with propidium iodide, Leica TCS SP5, Leica Microsystems, IL) and Scanning electron microscopy (SEM). In order to prepare liposomes for the latter, liposomes were placed on a coverslip and coated with Platinum/Palladium (Cressington sputter coater 208 HR, Cressington Scientific Instruments Ltd, UK). The surface topography of liposomes was visualized with scanning electron microscope (Zeiss Supra 40VP, CA, USA) in high-vacuum mode.

Drug release

Ciprofloxacin release from the liposomes were studied as described by Halwani M. *et al* 2008 with modifications [31]. The modified assay used in this study is similar to agar diffusion assay. However, UV absorbance at 272 nm was used to estimate ciprofloxacin concentration instead of the zone of inhibition of microbial growth due to the potential variations of antibiotic penetration efficiencies through agar, asymmetry of zone of inhibition and variations among the microorganism used in agar diffusion.

Known volumes of liposomes were added to 10ml of saline and incubated at 37^{0}C for 24h in a shaker (250rpm). The liposomal suspensions were withdrawn in defined time intervals, centrifuged (15000rpm, 10 min) and the absorbance of the supernatant was measured at 272nm (Infinite M200, Tecan Systems Inc., CA, USA) to estimate ciprofloxacin release . The liposome pellet was re-suspended in fresh saline (10ml) after each time interval to prevent possible antibiotic saturation.

After 24h of incubation, a sample was prepared as mentioned above and filtered (0.22µm). The quantification of farnesol released was assessed by HPLC assay as described above.

Microorganisms and growth conditions

Pseudomonas aeruginosa PAO1 was used throughout the study. The identity of the bacteria was confirmed with the commercially available API 20 E kit (Biomérieux, Mercy I'Etoile, France). All isolates were stored in multiple aliquots at -20^{0}C, after confirming their purity. Blood Agar (Sigma Aldrich, USA) and Brain Heart Infusion (BHI, Sigma Aldrich, USA) solutions were used for culturing *P. aeruginosa*.

Prior to each experiment, *P. aeruginosa* was subcultured on blood agar for 18 h at 37^{0}C. A loopful of the overnight bacterial growth was inoculated into BHI medium, and, incubated for 18h in an orbital shaker (80 rpm) at 37^{0}C. The resultant growth was harvested, washed twice in Phosphate Buffered Saline (PBS, pH 7.2) and resuspended. The concentration of *P. aeruginosa* was adjusted 1×10^7 cells/ml by spectrophotometry and confirmed by hemocytometric counting.

Biofilm Formation

P. aeruginosa biofilms were developed as described by Bandara et al [32] with some modifications. Commercially available pre-sterilized, polystyrene, flat bottom 96-well microtiter plates (BD Biosciences, California, USA) were used. Hundred µl of a standard cell suspension of bacteria (10^7organisms/ml) was prepared and transferred into the wells of a microtiter plate, and the plate was incubated for 1.5h (37^{0}C, 80 rpm) to promote microbial adherence to surface of the wells. After the initial adhesion phase, the cell suspensions were aspirated and each well was washed twice with PBS to remove loosely adherent cells. A total of 200µl of BHI was transferred to each well and the plate was incubated for 24 h (37^{0}C, 80 rpm), and wells washed twice with PBS to eliminate traces of the medium. The resultant biofilms were considered ready for experimental use.

Determination of minimum inhibitory concentration (MIC)

Planktonic phase

MIC was determined by a broth microdilution assay in accordance with the CLSI guidelines [33]. Briefly, bacterial cell suspensions (5×10^5 Cells/ml) were treated with ciprofloxacin or farnesol in a concentration gradient (two-fold) and incubated in a 96 well microtiter plate for 24h at 35^0C. At the end of the incubation, the optical density was measured by a spectrophotometer at 595 nm. The lowest concentration of the antibiotic or farnesol at which the bacteria demonstrated visible growth inhibition compared to the solvent control was considered the MIC of the antibiotic or farnesol against *P. aeruginosa*. The assay was performed in quadruplicates at three times.

Biofilm phase

P. aeruginosa biofilms were grown in sterile 96 well plates (BD Biosciences, USA) as described above. Biofilms were washed twice with PBS and ciprofloxacin or farnesol was administered in a concentration gradient (two fold). The plates were incubated for 24h at 37^0C and 80 rpm.

At the end of the incubation period, a XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) reduction assay was performed to quantify the viability of biofilms as described in following sections. The lowest concentration of the antibiotic or farnesol at which the bacteria demonstrate 80% of reduction of the viability compared to the solvent control is considered as the Minimum Biofilm Inhibitory Concentration (MBIC) of the antibiotic or farnesol against *P. aeruginosa*. The assay was performed quadruplicates three separate times.

In addition, as described in following sections, a crystal violet assay was performed at the end of the incubation to estimate the effect of farnesol on the biomass of the mature *P. aeruginosa* biofilms. The assay was performed in quadruplicates at three separate times.

Treatment of biofilms with liposomes

P. aeruginosa biofilms were developed in 96 well plates as described above. Freshly prepared liposomes (L_{con}, L_{cip}, L_{far} and $L_{cip+far}$) were added in a concentration gradient to the biofilms with BHI and incubated for another 24h (37^0C, 80rpm). At the end of this incubation, biofilms were washed twice with PBS and a XTT reduction assay was performed. Each experiment was conducted in quadruplicates on three different occasions.

XTT reduction assay

At the end of incubation of both test and control biofilms, a standard XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) reduction assay was performed as described by Bandara et al [34] to measure the viability of biofilms by means of bacterial cell metabolic activity. In brief, commercially available XTT powder (Sigma, MO, USA) was dissolved in PBS to a final concentration of 1 mg/ml. Then the solution was filter-sterilized (0.22 μm pore size filter) and stored at -70^{0}C. Freshly prepared 0.4 mM menadione solution was used for XTT reduction assay. Thawed XTT solution was mixed with menadione solution in a 20:1 (v/v) ratio immediately before the assay. Thereafter, PBS: XTT: Menadione in a 79:20:1 ratio were added into each well containing biofilms and incubated in the dark for 5 h at 37^{0}C. The color changes were measured with a microtiter plate reader (Infinite M200 microplate reader, TECAN US Inc, NC, USA) at 492nm.

Crystal violet assay

At the end of incubation of both test and control biofilms, a crystal violet assay was performed to quantify biofilm biomass. Biofilms were carefully washed twice with PBS and stained with a 1% crystal violet solution for 15 min at 25^{0}C without shaking. Wells were carefully washed three times with PBS to remove excess stain and air dried at room temperature. Thirty percent acetic acid was added to the wells containing stained biofilms and incubated for 20 min at 25^{0}C. The solution was transferred to a new well plate and the optical density was measured at 570nm.

Confocal Laser Scanning Microscopy (CLSM)

Biofilms were prepared on cover slips placed in flat bottom six well plates (Nunclon, Nunc, thermo Fisher scientific, USA) as described above. Pre-formed 24h biofilms were exposed to all 4 different liposomal preparations and incubated for another 24h at 37^{0}C in a shaker (80rpm). At the end of incubation, the prewashed films were stained with Live and Dead stain (Live/Dead BacLight Bacterial Viability kit, Invitrogen, Eugene, USA) [32]. The biofilm was then analyzed using confocal laser scanning microscopy

Transmission electron microscopy (TEM)

P. aeruginosa biofilms were prepared on ACLAR® film (Electron Microscopy Sciences, PA, USA) and treated with various liposomal preparations as described above. At the end of the incubation, biofilms were washed twice with PBS and fixed with an aldehyde mixture for 2-4h on ice (4% gluteraldehyde, 2% paraformaldehyde, 0.1M cacodylate, 2mM Ca and 4mM Mg). The samples were

then washed three times with 0.1M sodium cacodylate buffer (pH7.4, Electron Microscopy Sciences, PA, USA) for 15 min. Subsequently, the biofilms were fixed with reduced osmium (1:1 4% potassium ferrocyanide in 0.2M cacodylate buffer: 4% osmium tetroxide) in a microwave (100w, 3 sessions of 2 min on, 2 min off, two times). After fixation, the samples were washed 5 times with water for 10 min and dehydrated with ethanol (15 min each, 50%, 70%, and 95%, and 2×100% ethanol) and 100% acetone (15 min, two times). The samples were placed in a polymerization tray and infiltrated with resin (30%, 66% and 2×100% resin in 100% acetone) and polymerized for 2 days at 60^0C. The samples were then sectioned using a Leica UltraCut Ultramicrotome® (Leica Microsystems Inc. IL, USA), placed on grids and observed under an FEI Tecnai Transmission Electron Microscope® (FEI, Oregon, USA) at 80kV.

Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0). Mann—Whitney U-test was performed to compare the significant differences between corresponding controls and test samples of the *P. aeruginosa* biofilms. A P-value of less than 0.05 was considered statistically significant.

Results

Characteristics of liposomes

The entrapment efficiencies of ciprofloxacin were 60% in $L_{cip+far}$ and 61% in L_{cip}. Farnesol entrapment efficiencies were 91% in $L_{cip+far}$ and 93% in L_{far}. The diameters of L_{con}, L_{cip}, L_{far} and $L_{cip+far}$ were 2808.0±529.6nm, 677.8±46.6nm, 684.0±43.8nm and 536.8±21nm respectively. L_{con} were significantly larger than all three test liposomes and the difference in the diameters of test liposomes were insignificant ($P<0.05$). The zeta potentials of respective liposomes were -1.66mV, -3.27mV, +0.09mV and -0.11mV (Table 1) and polydispersity indices of respective liposomes were 0.086, 0.281, 0.391 and 0.213.

Morphology of liposomes

FM, TLM and SEM confirmed that the liposomes were spherical in shape and varied somewhat in size but were consistent with DLS measurements (control liposomes; L_{con}, Figure 1). FM images exhibited a fluorescently stained liposomal membrane and an unstained dark core (Figure 1).

Drug release

After 24h of drug release, 3.06% (18.04μg) of total encapsulated ciprofloxacin (589.14μg) was released from $L_{cip+far,}$ however, only 1.48% (13.27μg) of the total encapsulated ciprofloxacin (897.09μg) was released from L_{cip}. During a period of 24h, there was a continuous and a greater release of ciprofloxacin from $L_{cip+far}$ compared to L_{cip} (Figure 2).

After 24h, 1.81% (13.08μg) of total farnesol encapsulated (723.14μg) was released from $L_{cip+far}$. In contrast, 4.75% (35.13μg) of total farnesol encapsulated (738.91μg) was released from L_{far}.

Minimum inhibitory concentrations (MIC)

The MIC of ciprofloxacin was 0.125μg/ml for planktonic *P. aeruginosa* and MBIC was 16μg/ml for 24h developed *P. aeruginosa* biofilms.

Farnesol did not have any significant effects on the *P. aeruginosa* planktonic phase, even up to 175μg/ml. In contrast, the maximum reduction of metabolic activity as indicated by XTT readings (40% reduction compared to solvent controls, $P<0.05$) were observed at farnesol concentrations of 90μg/ml in established *P. aeruginosa* biofilms.

A crystal violet assay showed that concentrations of farnesol equal to or greater than 2.8μg/ml resulted in the greatest reduction in the biofilm biomass (45%, $P<0.05$) compared to its controls.

Effect of liposomes on the metabolism of established *P. aeruginosa* biofilms- XTT reduction assay

At the end of the 24h treatment of *P. aeruginosa* biofilms with various liposomal formulations, significant treatment effects were observed. The biofilms treated with $L_{cip+far}$ showed a significantly lower metabolic activity compared to L_{cip} and free ciprofloxacin. Only ≥0.128μg/ml ($P<0.05$) of ciprofloxacin concentrations delivered from $L_{cip+far}$ resulted in bacterial metabolic activity (80% reduction compared to control) equal to those observed in L_{cip} treated biofilms at ≥1.310μg ($P<0.05$) and, in contrast, 16μg/ml of free ciprofloxacin treated biofilms ($P<0.05$, Figure 3). Treatment with L_{far} and L_{con} did not exhibit any reduction of the *P. aeruginosa* biofilm metabolism compared to controls, as manifested by the XTT readings.

Effect of liposomes on *P. aeruginosa* biofilms - Confocal laser scanning microscopy

CLSM imaging also demonstrated the significant effect of the drug loaded liposomal preparations on *P. aeruginosa* pre-formed biofilms. Biofilms treated with liposomes containing either ciprofloxacin

(L_{cip}), farnesol (L_{far}), or both ($L_{cip+far,}$ Figure 4D, E, and F) exhibited a greater degree of structural disruption and a lower live: dead cell ratio compared to the dense, well-organized, undisturbed biofilm (Figure 4A) and biofilms treated with control liposomes (L_{con}, Figure 4C). It was clearly visible that the biofilms treated with $L_{cip+far}$ revealed the most significant disruption, exhibiting lessened biofilm mass/architecture and a significant proportion of dead cells compared to all other treatment combinations tested (Figure 4F). Interestingly, the biofilms treated with free ciprofloxacin demonstrated only isolated areas of cell death and were relatively undisrupted and appeared to be viable (Figure 4B) compared to L_{cip} and $L_{cip+far}$ treated biofilms (Figure 4E and F).

Figure 5 shows the longitudinal and horizontal sections of *P. aeruginosa* biofilms treated with various liposomal formulations. The biofilms treated with liposomal control, L_{con}, showed preserved dense biofilm architecture and the viability deeper bacterial cell layers. The dead and dying cells appeared only in the superficial cell layers of the biofilm (Figure 5A). Similarly, the L_{far} treated biofilm also exhibited a spatially oriented biofilm structure; however, regions of bacterial death were also noted (Figure 5B). The thickness of the dead cell layers in the L_{far} treated biofilm was greater than that of L_{con} treated biofilm and in some areas,the full thickness of the biofilm consisted of dead cells (Figure 5B). Compared to the L_{con} and L_{far} treated biofilms, the L_{cip} treated biofilms exhibited a greater disruption of the biofilm architecture and higher proportions of dead cells (Figure 5C). However, live cells were still existed in the deeper layers of the biofilm (Figure 5C). In contrast, the $L_{cip+far}$ treated biofilm showed the most significant quantities of dead bacteria compared to any of the other liposome treated biofilms. More importantly, the full thickness of the majority of the areas of the biofilm was filled with dead cells and the entire biofilm appeared dead (figure 5D).

Effect of liposomes on *P. aeruginosa* biofilms – Transmission electron microscopy

TEM imaging corroborated with the CSLM findings of the biofilms treated with various liposomal preparations. *P. aeruginosa* cells in the control, untreated biofilms appeared to be healthy with a rod shaped cell structure and dark intracellular materials. Cells were loosely packed and some clearly showed intact cell membranes and slimy secretions in their immediate environment. There were few dead cells (observed as clear cells), while some cells showed disintegration of cell membrane and cellular detritus (Figure 6A). Similarly, L_{con} treated biofilms also showed healthy heterogeneous bacteria with some dividing cells. Cell membranes were intact and no dead cells were visible (Figure 6B). Similar to the L_{con} treated biofilm, L_{far} treated biofilm also consisted of healthy bacteria with some pale colored/less condensed intracellular materials (Figure 6C). In

contrast, L_{cip} treated biofilms had higher numbers of dead cells/clear cells. The cell membranes of the majority of the cells were disintegrated and the cellular contents appear to be leaked out. Small vesicles were also present surrounding dead cells (Figure 6D). Most importantly, the $L_{cip+far}$ treated biofilms also exhibited the highest proportion of clear and partially ruptured cells. Some disintegrated cells contained cytoplasm filled vesicles. Cell debris of dead cells was distributed throughout the microscopic field (Figure 6E).

Discussion

The biofilm antibiotic resistance and the synthesis of various virulence determinants During *P. aeruginosa* biofilm formation are mediated by two major and chemically distinct quorum sensing systems; N-acetyl homoserine lactones and the 4-quinolones. Among all quinolones, only PQS (2-heptyl-3-hydroxy-4-quinolone, Pseudomonas Quinolone Signal, PQS) is isolated in broncho-alveolar lavage fluid in CF lungs suggesting the potential association of PQS with *P. aeruginosa* infections and subsequent inflammatory damage to host respiratory tissues in CF patients [26, 35, 36].

The role of PQS in *P. aeruginosa* biofilm development is well-known. PQS enhances *P. aeruginosa* biofilm formation by stimulating two known regulators of biofilm development; RhlR/C4-HSL and RpoS [37]. Also, PQS regulates the synthesis of extracellular DNA (eDNA) in *P. aeruginosa* biofilms. eDNA acts as a cell-cell interconnecting compound that predominantly maintain the 3D structure and architecture of *P. aeruginosa* biofilms [38]. eDNA is also isolated at very high concentrations in sputum samples of CF lungs (up to 20mg/ml), suggesting the existence of eDNA rich micro colonies of *P. aeruginosa* in CF lungs [39]. Alarmingly, eDNA enriched *P. aeruginosa* biofilms showed up to 640-fold more antibiotic resistance than planktonic cultures [40]. Importantly, PQS regulates the synthesis of various other extracellular proteases such as hydrogen cyanide and redox active phenazines like pyocyanin in *P. aeruginosa*[26, 41]. Pyocyanin, in turn, generates the reactive oxygen species (ROS) superoxide and hydrogen peroxide [42]. These ROS favor bacterial colonization by damaging various host cells by neutrophil apoptosis, induction of IL-8 and inhibition of the dual-oxidase based antimicrobial system in airway epithelia [43, 44]. Thus, pyocyanin producing *P. aeruginosa* strains are more difficult to clear from the lung by host immune reactions than non-pyocyanin producing *P. aeruginosa* strains [45]. In addition, PQS induces *P. aeruginosa* to enter into a metabolically less active state to cope external stresses such as antibiotics [46]. Due to its prominent role in *P. aeruginosa* biofilm development and maintenance, PQS appears as an attractive target for novel therapeutic strategies in pathogenic biofilm elimination by improving drug delivery.

A major QSM secreted by *Candida albicans*, *E,E*-farnesol, has been identified to significantly reduce PQS synthesis in *P. aeruginosa* [26]. In addition, farnesol is known to inhibit swarming of *P. aeruginosa*, most likely through inhibiting PQS synthesis [27]. Interestingly, using The Lubbock Chronic Wound Biofilm model, recent research revealed that farnesol at a concentration of 1,000µg/ml could completely suppress the development of *P. aeruginosa* biofilms [47]. Moreover, a role of farnesol in potentiating bacterial susceptibility to antibiotics has been recently unveiled [48]. Hence, we designed a novel drug delivery system, exploiting aforementioned anti-pseudomonas properties of farnesol, using well-established liposomes to co-deliver ciprofloxacin and farnesol simultaneously. The latter delivery system was expected to achieve superior elimination of mature *P. aeruginosa* biofilms through farnesol-mediated inhibition of PQS synthesis and ciprofloxacin-mediated killing of the bacteria. Furthermore, farnesol has very low aqueous solubility and is lipophilic, attributes making formulation in liposomes an attractive option.

Liposomes were originally developed for use in intravenous drug delivery systems. For localized target release of the antibiotic such as in managing lung infections, liposomes must be intact until reaching the desired site of infection and have slow and sustained drug release. The therapeutic use of liposomes for inhaled drug delivery has been recently studied [49-51]. Liposomes are expected to deliver antibiotics sufficiently and sustainably to the site of infection in the lung. In effect, when tobramycin is administered intratracheally in the form of liposomes, drug retention and antimicrobial activity in the lung are significantly enhanced [52-54]. To synthesize liposomes with substantial encapsulation of both ciprofloxacin and farnesol that exhibit a slow and sustained release, we experimented with various combinations of DPPC and cholesterol. The optimal ratio of these lipids for drug encapsulation and release was 4:1 DPPC: Cholesterol (data not shown). The resultant, drug-loaded multi lamellar liposome populations were heterogeneous as indicated by the polydispersity indices and ranged between 500-700nm in diameter. Zeta potential is a valuable physicochemical parameter that determines the stability of any nanosuspensions such as liposomes. The repulsion between liposomes with similar electric charges ensures easy redispersion of the particles and prevents their aggregation. Hence, extremely positive or negative zeta potential values are preferred[55, 56], i.e. a minimum zeta potential of ± 20 mV is desirable for combined electrostatic and steric stabilization of a nanosuspension [57] . However, all the multi lamellar liposomes prepared in our study demonstrated weak zeta potentials (between 0±5mV) and the tendency toward liposomal aggregation was confirmed through TLM imaging, where some liposomal aggregates were visible among individually dispersed liposomes. Thus, weak charge of liposomes may have influenced its stability, drug encapsulation and their adsorption to pathogens

[58]. Addition of surfactant may have added an advantage of dissolution of farnesol and increase the stability of liposomes. However, due to potential cytotoxic effects of various surfactants on *P. aeruginosa* biofilms and to simplify the liposomal composition, surfactants were not used [59]. Nevertheless, the liposomes reported here will be further optimized for a desirable zeta potential and stability in the future.

The relevance of the liposomal charge and its capability of penetration to the biofilms have been recently studied [60]. John et al showed that the penetration of oral and topical bacterial biofilms by charged (+ or -) liposomes was significantly higher than that of free drug [61-63]. In contrast, uncharged liposomes demonstrated more favorable interactions with planktonic *P. aeruginosa* [64]. In concurrence with these findings, our liposomes were weakly charged (+ or -) and thus expected to penetrate *P. aeruginosa* biofilms more effectively than free drug. The CLSM images presented in this study further supported the enhanced penetration of liposome into *P. aeruginosa* biofilms. Compared to free ciprofloxacin treated biofilms, L_{cip} and $L_{cip+far}$ exhibited significantly higher ratio of biofilm death, likely due to enhanced penetration. In particular, $L_{cip+far}$ treated biofilms showed bacterial cell death throughout the full thickness of the biofilms compared to other tested liposomal formulation exhibiting its properties of superior biofilm killing. TEM images were also consistent with these observations and L_{cip} and $L_{cip+far}$ killed more bacteria in *P. aeruginosa* biofilms compared to other liposomal preparations.

In the design of liposomes, farnesol was expected to be entrapped in the lipid bilayer due to its lipid solubility, whereas the water soluble ciprofloxacin was expected to be encapsulated in the core of the liposome. Farnesol demonstrated a very high efficiency of entrapment in both L_{far} and $L_{cip+far,}$ (over a 90% entrapment). However, the release of farnesol from $L_{cip+far}$ was 40% lower than that from L_{far} during a 24h period, suggesting that the presence of ciprofloxacin affects the release of farnesol. Drug release from liposomes is known to be influenced by the membrane composition and the biochemical properties of the encapsulated drug (charge, pK_as, drug stability, pH, etc.). Though the exact reason is yet to be unraveled, the presence of ciprofloxacin may have altered the liposomal stability resulting reduced farnesol release[65-67]. Similar to farnesol entrapment, ciprofloxacin was also entrapped in liposomes efficiently regardless of the presence of farnesol. However, ciprofloxacin showed constantly higher rates of release from $L_{cip+far}$ compared to L_{cip} throughout a 24h period, indicating that the presence of farnesol in the liposome increased the release of the antibiotic by two fold. Hence, from a drug release point of view, $L_{cip+far}$ was a preferred

antibiotic release system despite possessing similar entrapment efficiencies of farnesol and ciprofloxacin compared to L_{cip} and L_{far}.

The most important and unique features of these dually loaded liposomes were noted when delivered to mature *P. aeruginosa* biofilms. A significant reduction of the biofilm viability was noted at 0.128μg/ml ciprofloxacin released by $L_{cip+far}$. However, to achieve a similar efficiency in biofilm viability reduction, ten-fold more ciprofloxacin was needed in the form of L_{cip}. These results strongly indicate that $L_{cip+far}$ possesses an enhanced functional capability of killing established *P. aeruginosa* biofilms compared to liposomal ciprofloxacin (L_{cip}). It further confirms that the addition of farnesol to the liposomes significantly reduced the antibiotic dose needed to effectively eliminate biofilms. Furthermore, over 16μg/ml of free ciprofloxacin was needed to achieve a similar biofilm inhibition to the liposomal formulations indicating liposomal formulations offer superior anti-biofilm properties in comparison to free ciprofloxacin. Similarly, previous studies also showed superior biofilm inhibitory properties when active agents were co-delivered. For instance, adsorption of antibiotic loaded liposomes to zinc citrate particles produced solid supported vesicles and provided an additional inhibitory effect against *S. oralis* biofilms compared to liposomal encapsulated antibiotics without zinc citrate particles [68].

Co-delivery of more than one antimicrobial agent to target the site of infection via liposomes was hypothesized to provide a wide range of antibacterial effects with low drug toxicity as well as efficient drug release, even at sub-minimal concentrations For example, it was recently shown that co-encapsulation of bismuth with tobramycin increases the potential of *P. aeruginosa* killing, secretion of homoserine lactones, and reduces the bacterial adhesion [69]. In addition, bismuth EDT-tobramycin combination has been shown to disturb bacterial membrane integrity and biofilm formation [70, 71]. Interestingly, co-encapsulation decreased the toxic effect of bismuth to lung epithelium [31, 72]. In contrast, farnesol, the molecule used in our study, is known to be non-toxic for humans and possesses anticancer properties [73]. In fact, farnesol is a common ingredient used in colognes and fragrances [74], thus direct contact with skin is well-tolerable. Farnesol is well-metabolized by liver cells. Thus, the safety margin of liposomes loaded with farnesol and ciprofloxacin is expected to be greater compared to previously reported liposomal formulations. The proposed mechanisms of action of farnesol and ciprofloxacin loaded liposomes are presented in Figure 7. Further studies on the toxicity of these liposomal formulations need to be conducted. Nevertheless, a combination therapy of ciprofloxacin with farnesol in the form of liposomes, appear

to be a promising approach in eliminating devastating infections of *P. aeruginosa* such as in CF patients.

Summary

This study gives an important insight into a novel antipseudomonal biofilm strategy that synergistically combines microbial quorum sensing and bactericidal agents in a rationally designed delivery system for biofilms. The findings of this study confirmed that co-delivering farnesol and ciprofloxacin in liposomes allows for superior biofilm killing at significantly lower antimicrobial doses. The findings of this study support further investigation into this novel drug delivery system in managing *P. aeruginosa* infections in CF models.

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Legends

Table 1

The sizes, zeta potentials and polydispersity indices of various liposomal preparations

The size of the liposomes are measured by their diameter.

Figure 1

Qualitative analyses of liposomes with fluorescent microscopy, transmission light microscopy and scan electron microscopy

A. Liposomes under transmission light microscopy; note the circular and multi lamellar nature of liposomes. B. and C. Liposomes stained with propidium iodide under fluorescent microscopy; the liposomes were circular and the lipid walls of liposomes were clearly stained with fluorescent dye while the core appeared unstained. D. and E. Liposomes under scan electron microscopy; note the globular topography of liposomes.

Figure 2

Release of ciprofloxacin from L_{cip} and $L_{cip+far}$ for 24h

Total ciprofloxacin released from $L_{cip+far}$ was higher than from L_{cip}. Both liposomes released more ciprofloxacin than their respective biofilm inhibitory concentrations.

Figure 3

Minimum biofilm inhibitory concentrations of ciprofloxacin when administered in different forms

The ciprofloxacin concentration needed for an inhibition of *P. aeruginosa* biofilms was significantly lower when administered as L_{cip} (1.31µg/ml) and $L_{cip+far}$ (0.128µg/ml) compared to free ciprofloxacin (16µg/ml). Most significant effect was observed with $L_{cip+far}$ ($P<0.05$) and the concentration of ciprofloxacin required in the form of $L_{cip+far}$ was significantly lower than L_{cip} ($P<0.05$). * indicates significant differences.

Figure 4

Effect of liposomes on *P. aeruginosa* biofilms - Confocal laser scanning microscopy (magnification × 40) (stained using a LIVE/DEAD BacLight bacterial viability kit; Invitrogen); Live cells are stained in green and dead cells in red.

(A) *P. aeruginosa* 24h control biofilm; (B) *P. aeruginosa* biofilm treated with free ciprofloxacin 0.128µg/ml. (C) *P. aeruginosa* biofilm treated with L_{con}. (D) *P. aeruginosa* biofilm treated with L_{far}. (E) *P. aeruginosa* biofilm treated with L_{cip} (Final ciprofloxacin concentration was 0.128µg/ml (F) *P. aeruginosa* biofilm treated with $L_{cip+far}$ (Final ciprofloxacin concentration was 0.128µg/ml.). L_{cip}, L_{far} and $L_{cip+far,}$ (Figure 4D, E, and F) exhibited greater degree of structural disruption, lower live: dead cell ratio compared to dense, spatially oriented control biofilms or biofilms treated with L_{con} (Figure 4A, and C). $L_{cip+far}$ treated biofilms demonstrated the most significant disruption. Note the scanty biofilm architecture, and a significant proportion of dead cells compared to all other samples (Figure 4F). Free ciprofloxacin treated biofilm was undisrupted and appeared to be alive despite few isolated areas of cell death (Figure 2B) compared to L_{cip} and $L_{cip+far}$ treated biofilms (Figure 4E and F) .

Figure 5

The depth of liposomal activity in *P. aeruginosa* 24h biofilms - Confocal laser scanning microscopy (magnification × 40) (stained using a LIVE/DEAD BacLight bacterial viability kit; Invitrogen); Live cells are stained in green and dead cells in red.

(A). L_{con} treated *P. aeruginosa* biofilm. Note the 3D biofilm architecture and the distribution of live and dead cells along the thickness of the biofilm (B). L_{far} treated *P. aeruginosa* biofilm. Note the islands of dead bacteria and thinning of the biofilm (C). Lcip treated *P. aeruginosa* biofilm. Note the grater proportions of dead cells compared to (A) and (B) and thinning the biofilm (D). $L_{cip+far}$ treated *P. aeruginosa* biofilm. Biofilm is completely dead both superficially and throughout the thickness and only few live cells are visible.

Figure 6

Cellular level effects of liposomes on 24h *P. aeruginosa* biofilms – Transmission electron microscopy (scale 500nm)

(A). Control *P. aeruginosa* 24h biofilm. Note the healthy, rod shaped, loosely packed cells with dark intracellular materials, intact cell membrane and slimy secretions in its immediate environment. Few dead cells appeared as clear cells. (B) L_{con} treated 24h *P. aeruginosa* biofilms. Note the healthy heterogeneous bacteria with some dividing cells with intact cell membranes. (C) L_{far} treated *P. aeruginosa* 24h biofilm. Note the similarity with (A), however, cells appear slightly larger than those in control biofilm. (D) L_{cip} treated *P. aeruginosa* 24h biofilms. Higher numbers of dead cells/clear

cells and disintegrated cell membranes were noted. Cellular contents appear leaked. Dead cells were surrounded by small vesicles. (E) $L_{cip+far}$ treated *P. aeruginosa* 24h biofilms. Note the clear and partially ruptured cells and cellular debris throughout the microscopic field. Some disintegrated cells contained cytoplasm filled vesicles.

Figure 7

Proposed probable mechanisms and summary of *P. aeruginosa* biofilm disruption by Farnesol and ciprofloxacin loaded liposomes

Liposome type	Diameter ±SD (nm)	Zeta potential (mV)	Polydispersity Index
L_{cip}	677.8±46.6	-3.27	0.281
$L_{cip+far}$	536.8±21.0	-0.108	0.213
L_{far}	684.0±43.8	0.086	0.391
L_{con}	2808.0±529.6	-1.66	0.086

Table 1

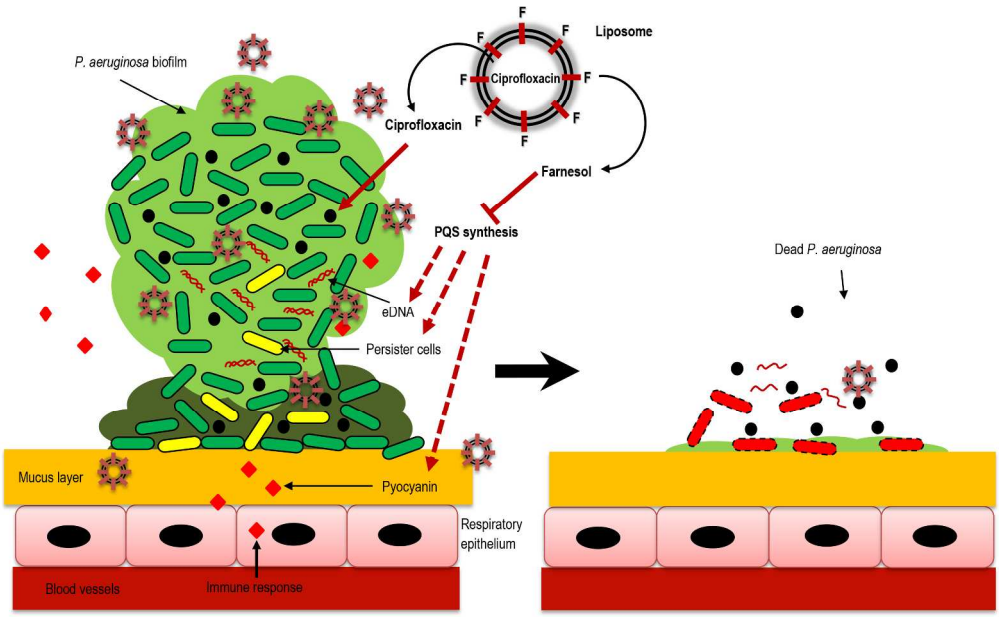

Table of contents graphic
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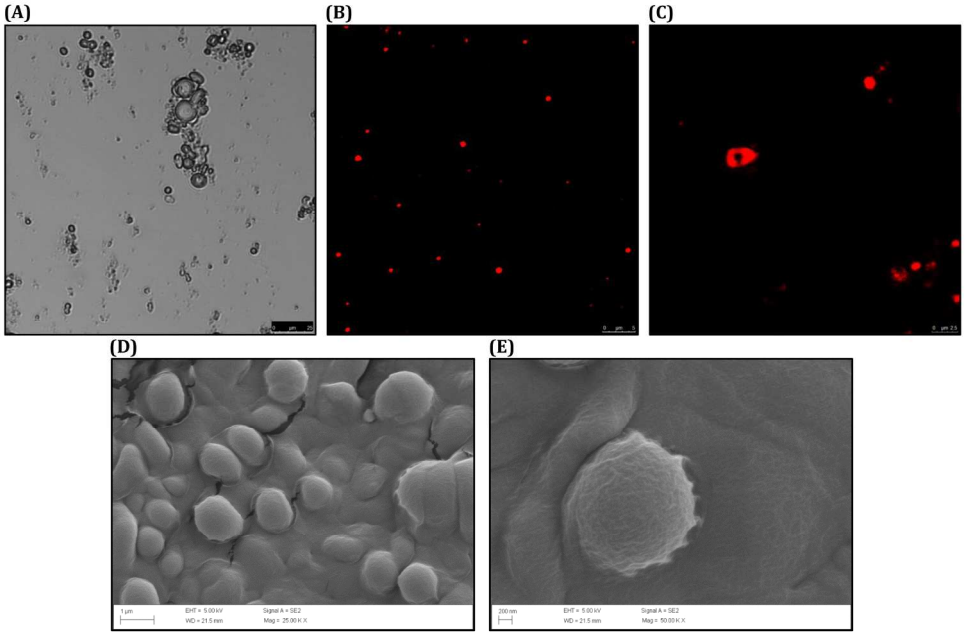

Figure 1
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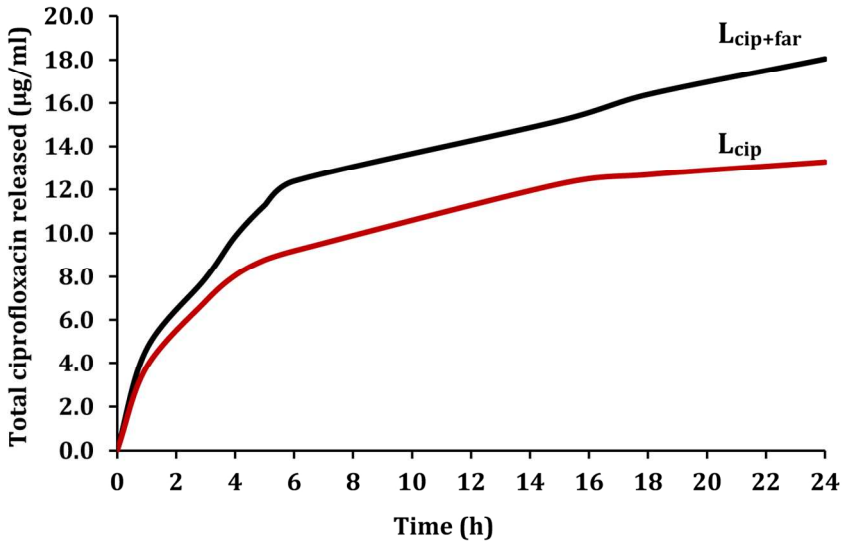

Figure 2
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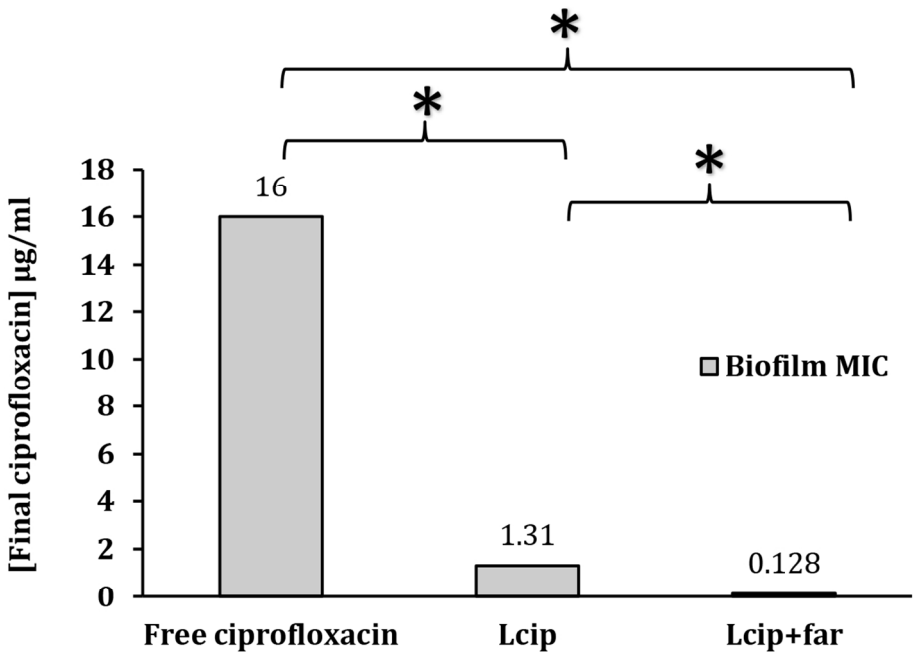

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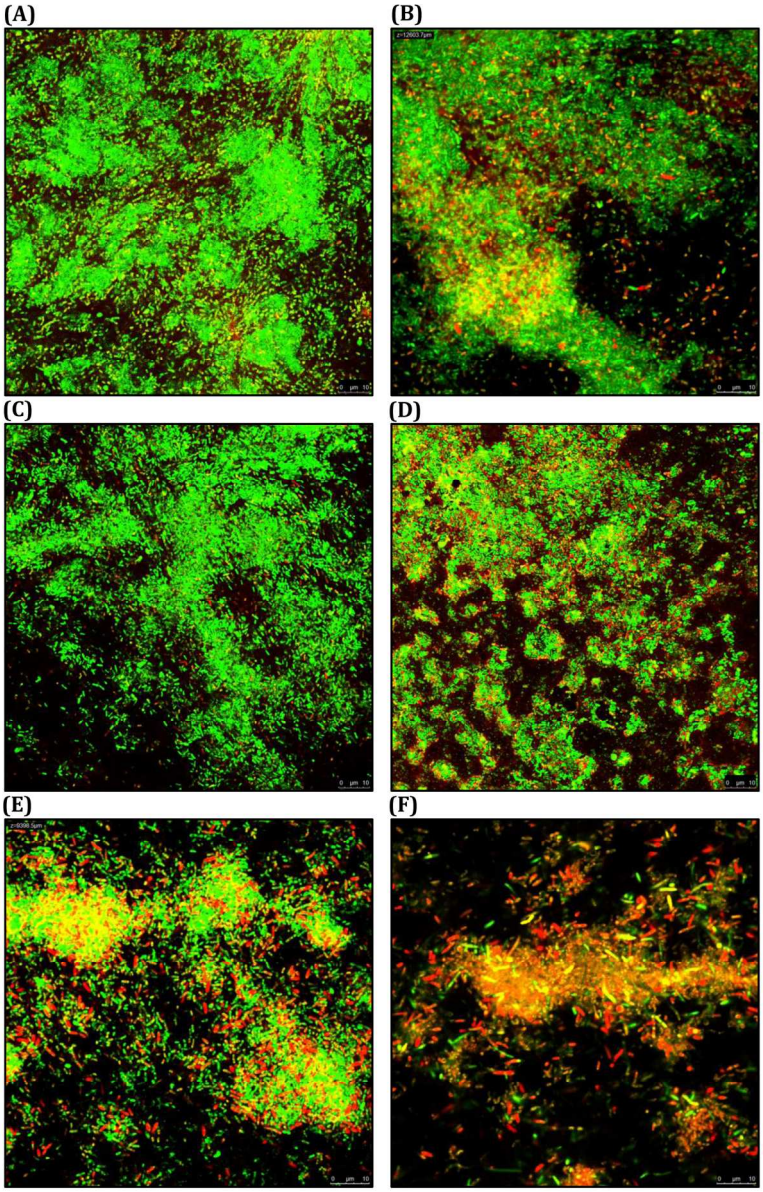

Figure 4
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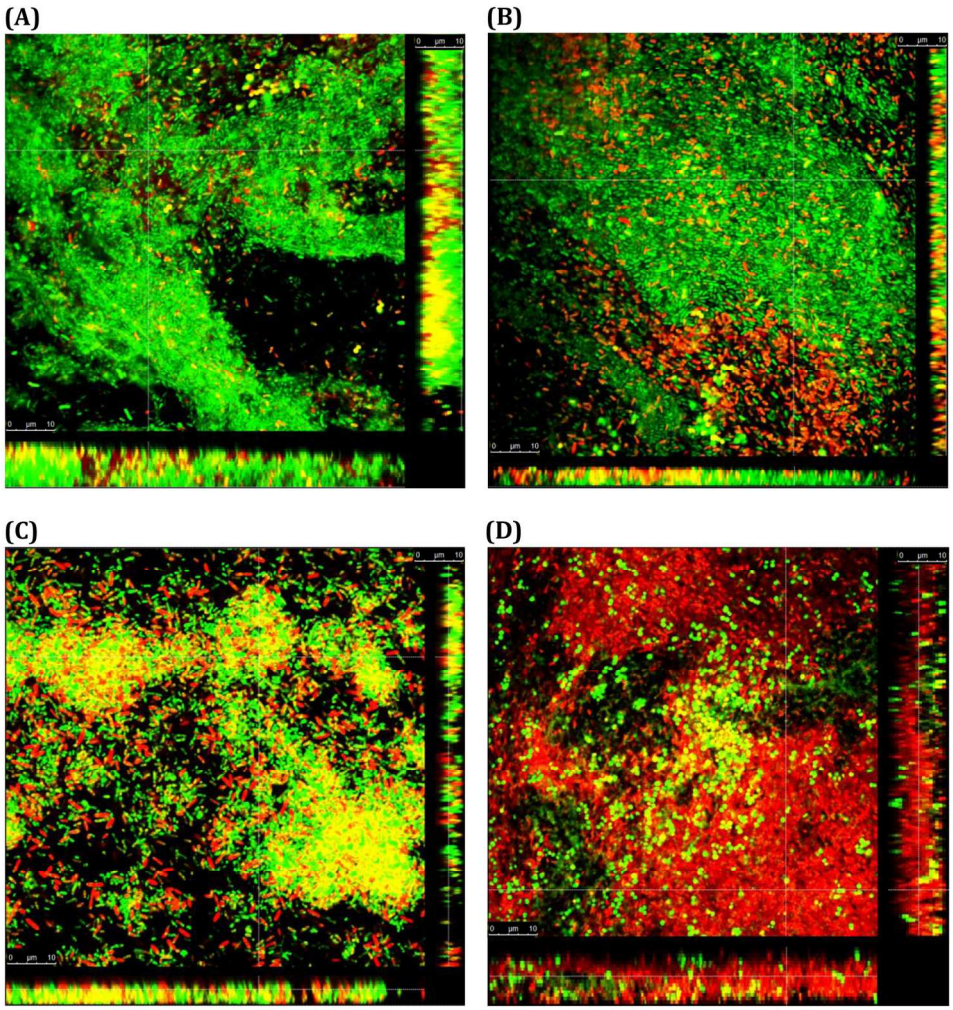

Figure 5
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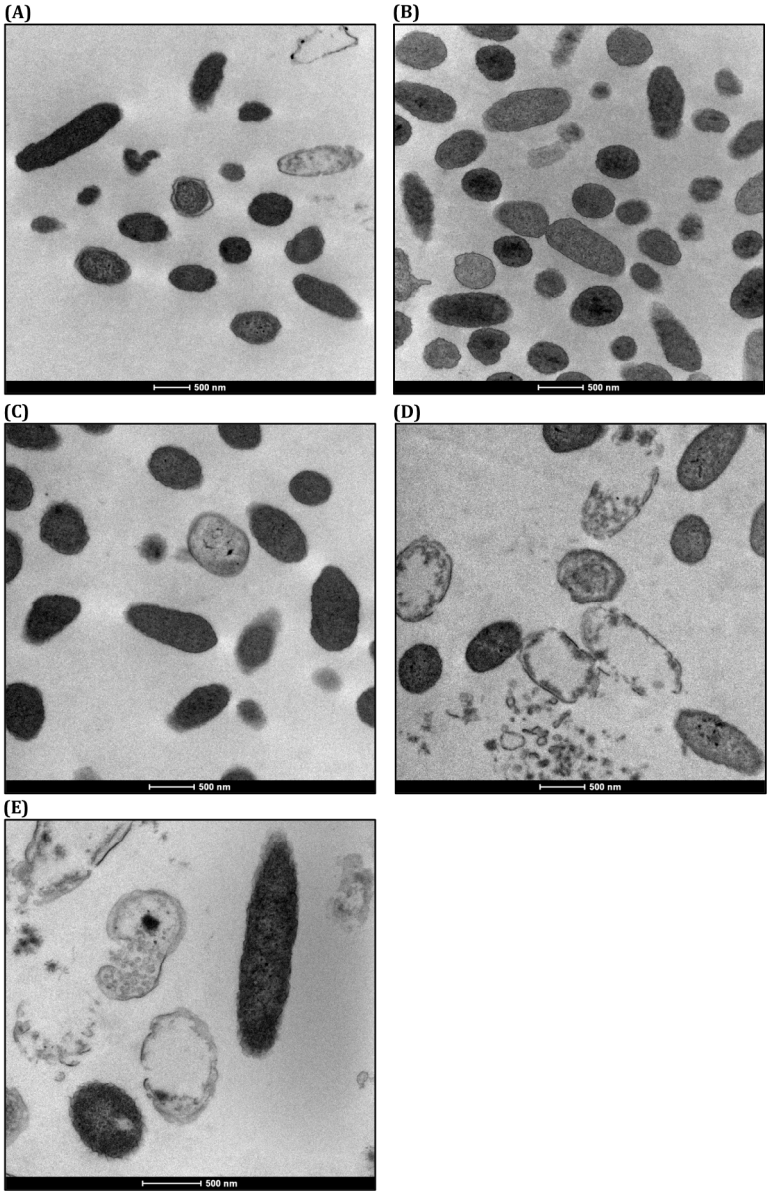

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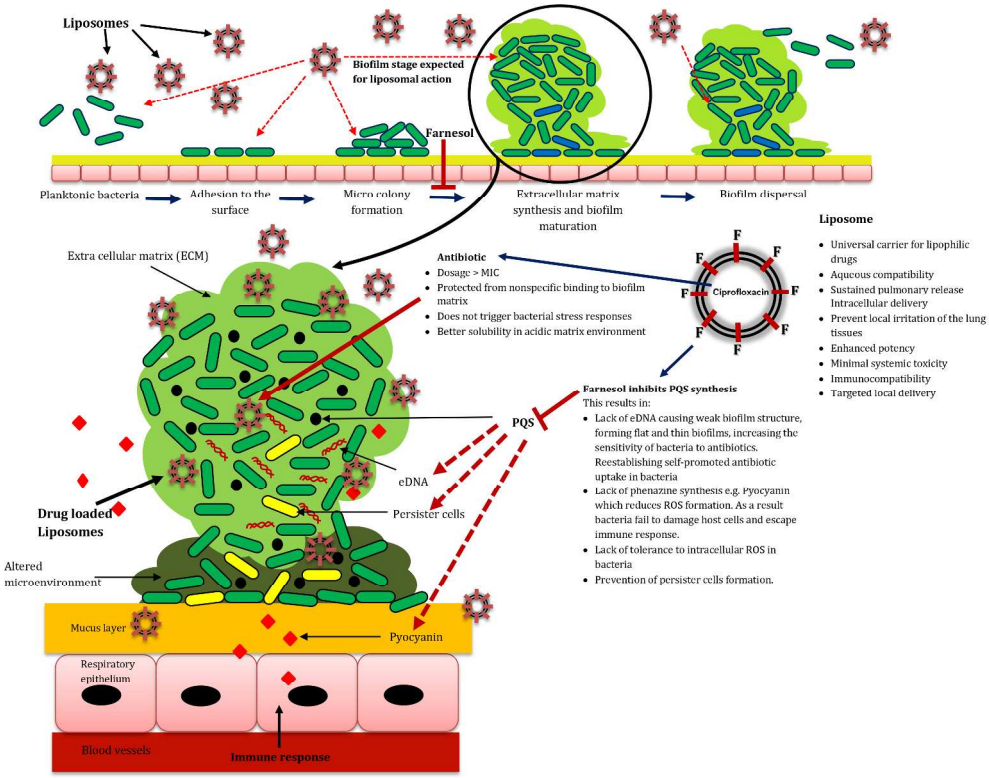

Figure 7
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